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Effects of glycosylation on antigenicity and immunogenicity of classical swine fever virus envelope proteins

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ABSTRACT

Classical swine fever virus (CSFV) harbors three envelope glycoproteins (E^{rns}, E1 and E2). Previous studies have demonstrated that removal of specific glycosylation sites within these proteins yielded attenuated and immunogenic CSFV mutants. Here we analyzed the effects of lack of glycosylation of baculovirus-expressed E^{rns}, E1, and E2 proteins on immunogenicity. Interestingly, E^{rns}, E1, and E2 proteins lacking proper post-translational modifications, most noticeable lack of glycosylation, failed to induce a detectable virus neutralizing antibody (NA) response and protection against CSFV. Similarly, no NA or protection was observed in pigs immunized with E1 glycoprotein. Analysis of E^{rns} and E2 proteins with single site glycosylation mutations revealed that detectable antibody responses, but not protection against lethal CSFV challenge is affected by removal of specific glycosylation sites. In addition, it was observed that single administration of purified E^{rns} glycoprotein induced an effective protection against CSFV infection.

Introduction

Classical swine fever (CSF) is a highly contagious and often fatal disease of swine. The etiological agent, classical swine fever virus (CSFV), is an enveloped virus that is classified as a Pestivirus within the family Flaviviridae (Fauquet et al., 2005). The CSFV genome is a positive sense single-stranded RNA that contains a single open reading frame that encodes an approximately 3900-amino-acid polyprotein. This polyprotein is co- and post-translationally processed by host and viral proteases to yield 11 to 12 final cleavage products (NH_2–Npro–C–E^{rns}–E1–E2–p7–NS2–NS3–NS4A–NS4B–NS5A–NS5B–COOH) (Lindenbach et al., 2007). There are three envelope glycoproteins in the CSFV virion, E^{rns}, E1, and E2 (Thiel et al., 1991). E^{rns} is loosely associated with mature virions (Thiel et al., 1991), it possesses ribonuclease activity (Rumenapf et al., 1993), and is heavily glycosylated with carbohydrate moieties at seven glycosylation sites contributing to nearly half of the molecular mass of the protein (Branza-Nichita et al., 2004; Sainz et al., 2008). E1 is a small 33 kDa protein that forms heterodimers with E2 protein (Weiland et al., 1990) and contains three glycosylation sites (Fernandez-Sainz et al., 2009; Thiel et al., 1993). E2 is a major determinant of CSFV virulence (Risatti et al., 2005; Van Gennip et al., 2004) and is involved in virus attachment and entry into target cells (Hulst and Moormann, 1997). E2 protein contains one putative *O*-linked glycosylation site and six *N*-linked glycosylation sites (Risatti et al., 2007; Thiel et al., 1991; van Rijn et al., 1994). E2 is the major CSFV immunogen and has been used to develop subunit vaccines. These vaccines induce protection against CSFV although the onset of immunity is significantly delayed relative to live attenuated vaccines (Moormann et al., 2000).

The effects of glycan moieties found in these envelope proteins on induction of host humoral responses and protection against CSFV are not well understood. For many enveloped viruses (e.g. hepatitis C virus and human immunodeficiency virus) patterns of envelope protein glycosylation have been shown to influence the number of available epitopes and to modulate immune recognition of antigens, affecting the humoral immune response (Fournillier et al., 2001). In the case of hepatitis C virus (HCV), carbohydrate moieties on viral envelope proteins modulate neutralizing activity. Using HCV infectious particles, it was observed that removal of single *N*-linked glycans at sites N1, N2, N4, N6 and N11 from E2 protein rendered mutant viruses highly sensitive to neutralization by antibodies from HCV-seropositive patients (Helle et al., 2010). These data suggest that those glycans reduce the

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accessibility of neutralizing antibodies to cognate epitopes in E2 glycoprotein. Mutation of the fourth glycosylation site in HCV E1, regarded as a poor immunogenic protein, significantly enhanced the anti-E1 humoral response in terms of both seroconversion rates and antibody titers (Fournillier et al., 2001). In the case of human immunodeficiency virus (HIV) gp120 envelope glycoprotein, proper expression of conformational epitopes is dependent on the integrity and presence of carbohydrate moieties (Haigwood et al., 1992), since fully glycosylated gp120 was found to present neutralizing epitopes more effectively than the nonglycosylated form of the envelope protein (Haigwood et al., 1992). Nevertheless, these glycan moieties also have been shown to play a role in the masking of viral neutralizing epitopes (Back et al., 1993), leading to the "glycan shield" concept that implicates carbohydrates in HIV neutralizing antibody resistance (Wei et al., 2003). Similarly, glycosylation of envelope proteins affects immunogenicity of Porcine Reproductive and Respiratory Syndrome virus (PRRSV). Three putative N-linked glycosylation sites (N34, N44, and N51) are located on the glycoprotein 5 (GP5) ectodomain of PRRSV, where a major neutralization epitope also exists. Mutations of residue N44 did not result in infectious progeny, whereas viruses carrying mutations at N34, N51, and N34/51 exhibited enhanced sensitivity to neutralization by wild-type PRRSV-specific antibodies. Furthermore, inoculation of pigs with the mutant viruses induced significantly higher levels of neutralizing antibodies against the mutant as well as the wild-type PRRSV, suggesting that the loss of glycan residues in the ectodomain of GP5 enhances both the sensitivity of these viruses to in vitro neutralization and the immunogenicity of the nearby neutralization epitope (Ansari et al., 2006).

In previous studies, we have observed that changes in the glycosylation patterns of CSFV envelope proteins indeed affected virus virulence and viability (Fernandez-Sainz et al., 2009; Risatti et al., 2007; Sainz et al., 2008). Those studies showed that substitutions of specific N residues to A, in the context of *N*-glycosylation consensus sequence N-X-S/T (Kornfeld and Kornfeld, 1985), within CSFV E^{rns} (N269A), E1 (N594A and N500A/N513A), or E2 (N116L) glycoproteins yielded attenuated viruses that were also immunogenic, inducing an effective protection against challenge with virulent CSFV as early as day 3 post-inoculation. Notably, the rest of the single glycosylation site mutant viruses were virulent in swine, while complete lack of glycosylation of these proteins in the context of an infectious cDNA clone was deleterious for CSFV (Fernandez-Sainz et al., 2009; Risatti et al., 2007; Sainz et al., 2008). Although significant, these observations limited the study of glycosylation and its effect on antigenicity and/or immunogenicity of CSFV envelope proteins. To overcome that limitation, we carried out comparative studies in swine inoculated with baculovirus-expressed CSFV BICv (strain Brescia infectious clone virus; Risatti et al., 2005) envelope proteins to address the role of glycosylation on the induction of humoral immune response and protection against the virus. Data presented here show that glycosylated forms of E^{rns} and E2 proteins induced virus neutralizing antibodies in immunized swine and conferred an effective protection against CSFV. In contrast, animals immunized with glycosylated E1 protein did not develop a detectable NA response and succumbed when exposed to the virulent virus. Interestingly, complete lack of immunogenicity was observed when pigs were immunized with nonglycosylated forms of the envelope proteins resulting from improper post-translational modifications of E^{rns}, E1, and E2 proteins. When assessing the effect on immunogenicity of individual glycosylation sites, it was observed that the removal of single sites from E^{rns} or E2 proteins did not affect their ability to induce a protective immunity against CSFV, although it had an effect on the induction of the NA response. Results described here show that glycosylation plays a major role in the immunogenicity of CSFV envelope proteins, most likely an effect linked to the correct conformation of E^{rns}, E1, and E2 proteins.

Results

Expression of CSFV envelope proteins

Recombinant baculoviruses harboring CSFV envelope protein genes, with and without sequences encoding for signal peptides (Fig. 1), were used to infect Sf9 cells and protein expression was analyzed by

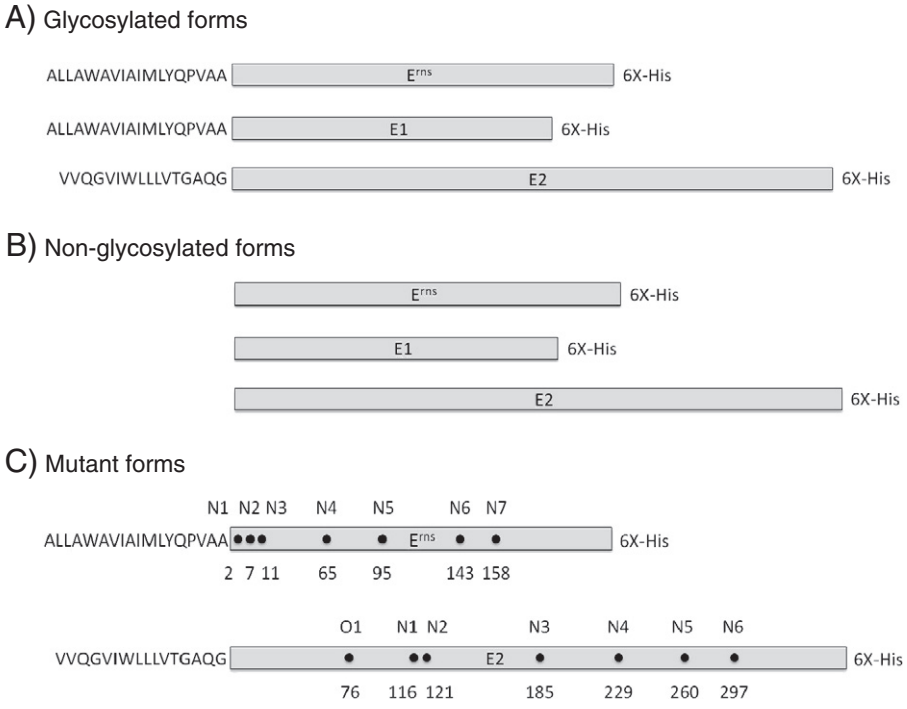

Fig. 1. Schematic representation of classical swine fever virus envelope proteins expressed in baculovirus. Glycosylated forms of the proteins were expressed with signal peptides encompassing the last 18 amino acids of Core protein (E^{rns} and E1) or the last 16 amino acids of E1 protein (E2) (A). Nonglycosylated forms of the proteins were expressed without signal peptides (B). Single site glycosylation mutant forms of E^{rns} and E2 proteins were generated by site-directed mutagenesis using wild-type genes as targets. Mutations of amino acid residues at indicated glycosylation sites within E^{rns} and E2 proteins (N and O) resulted in N to A substitutions in the consensus N-X-T/S (*N*-glycosylation) or P to A (*O*-glycosylation) (C). E2 was expressed without the trans-membrane domain. All proteins were expressed with poly-histidine tags (6× His) for detection and purification purposes.

Western blot. As shown in Fig. 2, glycosylated and nonglycosylated forms of E^{rns}, E1, and E2 were expressed and recognized by the anti-His C-terminus antibody. Glycosylated forms of the proteins showed the expected molecular sizes; E^{rns} 48 kDa, E1 31–32 kDa, and E2 55 kDa (Moser et al., 1996; Ruggli et al., 1995; Rumenapf et al., 1993). For the glycosylated E1 protein a second band of approximately 26 kDa was also detected (Fig. 2) that may represent different degrees of glycosylation (Fournillier et al., 2001). The electrophoretic mobility of E^{rns}, E1, and E2 expressed without signal peptides was found to be compatible with that of deglycosylated forms of the proteins that were expressed with signal peptides and previously treated with glycosidase PNGaseF. The molecular weights of nonglycosylated E^{rns}, E1, and E2 proteins were approximately 25 kDa, 21 kDa, and 41 kDa, respectively, and corresponded to their predicted mass calculated from the amino acid composition, indicating the absence of proper glycosylation (Fig. 2) (Ruggli et al., 1995).

Recombinant baculoviruses harboring E^{rns} and E2 mutated genes encoding for single N to A substitutions at *N*-glycosylation sites (N-X-T/S) and at the predicted *O*-glycosylation site in E2 protein were used to infect Sf9 cells. The differences in electrophoretic mobility observed among expressed E^{rns}, and E2 mutated proteins (Supplementary Fig. S1) corresponded to the differences observed in lysates from SK6 cells infected with glycosylation mutant viruses (Risatti et al., 2007; Sainz et al., 2008).

Immunogenicity of CSFV envelope proteins

To investigate the effect of modified forms of envelope proteins on the immunogenicity and protective efficacy against CSFV, pigs were inoculated with purified glycosylated or nonglycosylated forms of the CSFV envelope proteins. Animals were boosted at days 28, 42, and 56 after the first inoculation, and challenged IN one week after the last inoculation with a virus (BICv) derived from an infectious clone of the highly virulent isolate Brescia (Risatti et al., 2005). Pigs vaccinated with glycosylated E^{rns} and E2 were protected and survived challenge without showing clinical signs of the disease (Table 2). Only a mild and transient increase in body temperature was observed in two out of four animals inoculated with glycosylated E2 protein. Conversely, animals inoculated with nonglycosylated forms of E^{rns} and E2 proteins succumbed to the challenge, and showed signs of the disease indistinguishable from those observed in control animals. These data indicate that lacking proper post-translational modifications, most noticeable lack of glycosylation, significantly affects immunogenicity of E^{rns} and E2 proteins. Along with those observations, viremia in challenged animals immunized with nonglycosylated forms of E^{rns} and E2 was indistinguishable from viremia observed in control pigs (Figs. 3A and B). All these animals succumbed to infection no later than 14 dpc. Immunization with E^{rns} or E2 glycoproteins elicited a response that resulted in virus titers in blood approximately 100-fold lower relative to control pigs (Figs. 3A and B). Clearance of challenging virus was observed by 21 dpc in the E^{rns} glycoprotein group and by 14 to 21 dpc in the E2 glycoprotein group. Overall, data suggest that correct topology and structure of E^{rns} and E2 glycoproteins is necessary for eliciting an effective immune response against CSFV.

Pigs immunized with glycosylated or nonglycosylated form E1 protein were not protected against BICv (Table 2), developing CSF signs characterized by anorexia, depression, and an onset of fever by 3–4 dpc, ultimately succumbing to the infection within 8 to 14 dpc. E1 protein administered as purified protein does not induce protection against CSFV.

Antibody response in immunized pigs

The ability of glycosylated and nonglycosylated forms of E^{rns} and E2 proteins to induce antibodies in swine was tested by ELISA and seroneutralization following immunization.

Using ELISA, only animals immunized with glycosylated forms of E^{rns} and E2 proteins showed an antibody response (Fig. 4A). Similarly, neutralizing antibodies titers (NA) measured against BICv showed that immunization with nonglycosylated forms of E^{rns} or E2 did not elicit detectable NAs (Fig. 4B), correlating with the lack of immunogenicity induced in swine by these forms of the proteins. ELISA and NA data suggest that lack of proper conformation of E^{rns} and E2 glycoproteins considerably affects the exposure of critical epitopes.

NAs were induced by immunization with glycosylated forms of the proteins (Fig. 4B), correlating with the observed induction of protection against BICv. The NA response induced by glycosylated E2 occurred earlier than the response in pigs immunized with glycosylated E^{rns}. NA titers of 1:500 or higher were detected in serum samples from all animals at day 7 after the second dose of glycosylated E2 (Fig. 4B). A sustained antibody response was observed in this group of pigs until challenge, when NA titers in all the animals were detected above 1:2000. NA titers in pigs immunized with glycosylated E^{rns} were detected in one animal (#79 = 1:128) at day 7 after receiving a second dose of the protein, whereas a uniform response was observed in all pigs only by 14 days post-third immunization (Fig. 4B). Notably, NA titers in this group of animals were 1:64 by the time of challenge, and in spite of rather reduced E^{rns} antigenicity compared with that of E2, the induced antibody response precluded the appearance of CSF signs in these animals.

Immunogenicity of E^{rns} glycoprotein

The finding that purified glycosylated E^{rns} elicited low levels of NA titers but induced protection against challenge with BICv after four immunizations prompted analysis of whether or not this protein was still immunogenic after a fewer number of inoculations. Three different groups of pigs were intramuscularly immunized with one, two or three doses of E^{rns} glycoprotein at 14 day intervals (Fig. 5) and challenged at 21 days after the last inoculation. Interestingly, protection against challenge was achieved even when pigs received only one dose of E^{rns} (Table 3). Neither E^{rns} antibodies nor virus neutralizing antibodies against CSFV were detected in sera collected from these animals (Fig. 5C), although these pigs seroconverted by day 7 after challenge (data not shown). Animals that received 2 or 3 doses of E^{rns} presented low NA titers at challenge (Figs. 5A and B) and were clearly seropositive by ELISA. These data suggest either a low sensitivity of the antibody detection test used to quantify NAs, or additional yet unidentified immune mechanisms (i.e. non-neutralizing antibodies), are contributing to protection against CSFV induced by glycosylated E^{rns}.

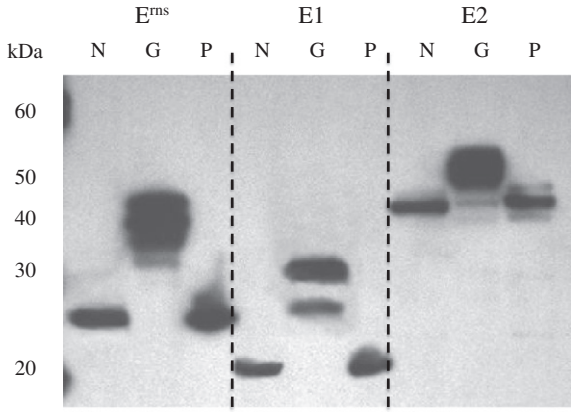

Fig. 2. Western blot analysis of baculovirus-expressed nonglycosylated (N), glycosylated (G), or glycosylated PNGase F-treated lysate (P) forms of CSFV BICv (Risatti et al., 2005) envelope proteins E^{rns}, E1, and E2. Lysates from Sf9 cells inoculated with recombinant baculoviruses were separated under denaturing conditions in 10% Bis–Tris gels followed by detection with anti-his C-term antibody (Invitrogen).

Table 1
Sequences of the oligonucleotide primers used for synthesis of glycosylated and nonglycosylated forms of E^{rns}, E1, and E2 proteins.

Primer name	Sequence[a]
E^{rns} nonglycosylated forward	5′ CACCATGGAAAATATAACTCAA 3′
E^{rns} glycosylated forward	5′ CACC*ATGGCCCTATTGGCATGGGCAGTGATAGCAATTATGTTATACCAACCTGTTGCAGCC*GAAAATATAACTCAA 3′
E^{rns} reverse	5′ TTAGTGATGGTGATGGTGATGGGCATAGGCACCAAA 3′
E1 nonglycosylated forward	5′ CACCATGCTATCACCTTATTGT 3′
E1 glycosylated forward	5′ CACC*ATGGCCCTATTGGCATGGGCAGTGATAGCAATTATGTTATACCAACCTGTTGCAGCCC*TATCACCTTATTGT 3′
E1 reverse	5′ TTAGTGATGGTGATGGTGATGGCCTTGTGCCCCAGT 3′
E2 nonglycosylated forward	5′ CACCATGCGGCTAGCCTGCAAG 3′
E2 glycosylated forward	5′ CACC*ATGGTCGTGCAAGGTGTGATATGGCTGTTACTGGTAACTGGGGCACAAGGC* CGGCTAGCCTGCAAG 3′
E2 reverse	5′ TTAGTGATGGTGATGGTGATGTTCTGCGAAGTAATC 3′

[a] Signal peptides shown in italics; his-tag sequences shown underlined.

Antibody response in pigs immunized with single glycosylation site mutated E^{rns} and E2 glycoproteins

Since modified E^{rns} and E2 lacking proper glycosylation abrogated the antibody and the protective response in inoculated pigs, we assessed if any of the individual glycosylation sites present in these proteins were critical in the induction of the antibody response. Animals were immunized with single site glycosylation mutated E^{rns} protein or E2 protein (Supplementary Fig. S1) and challenged with BICv as described above. Sera obtained from these animals were tested for the presence of antibodies.

No detectable NAs were present in sera from pigs that received mutated forms of E^{rns} protein. However, sera from all these animals tested positive by ELISA 7 days after receiving a second dose of the proteins (data not shown).

NAs were detected in sera from pigs immunized with single site glycosylation mutated forms of E2 protein by 7 days after the second inoculation (Fig. 6). Sera from pigs immunized with O1, N1, N2, N3, and N4 exhibited significantly lower NA titers compared to sera from animals immunized with E2 protein (Fig. 6), whereas mutations at N5 and N6 did not affect E2 immunogenicity (Fig. 6). Overall, most of the E2 single glycosylation mutations diminished the antigenicity of E2 protein.

Protection elicited by single glycosylation site mutated E^{rns} and E2 glycoproteins

The contribution of single glycosylation sites in E^{rns} and E2 proteins in conferring protection against CSFV was assessed by challenging immunized pigs (see above) with virulent BICv. While non-immunized control pigs succumbed to the lethal infection, all animals immunized with mutated forms of either glycoprotein survived the challenge and were protected against clinical disease after four inoculations, with the exception of pigs immunized with E^{rns} N3, E^{rns} N7, and E2 N2 proteins; these animals exhibited elevated body temperatures for more than 6 days (Table 4), a temporary loss of appetite and mild depression. Viremia was detected in all groups of immunized animals and for the same approximate length of time. Virus titers in animals immunized with mutated forms of E^{rns} or E2 tended to be higher than virus titers detected with native forms of the proteins. Nonetheless, in all cases, virus titers were significantly lower in animals immunized with different forms of E^{rns} or E2 than in mock-vaccinated animals (Figs. 7A and B).

Recombinant E2 single glycosylation mutant viruses have differential sensitivity to NAs raised against glycosylated E2 protein

The finding that immunization with mutated forms of E^{rns} and E2 elicited differential NA responses against BICv (Fig. 6) prompted the opposite analysis, where the neutralizing activity of sera raised against glycosylated E2 was determined against recombinant E2 glycosylation mutant viruses (Risatti et al., 2007). E2N1v, a mutant virus harboring a N116A substitution in E2, was significantly more resistant to neutralization by sera raised against glycosylated E2 than BICv (NAb titers of 220 vs. 603 respectively, $p<0.05$, *t*-test). Meanwhile E2N4v, a mutant virus harboring a N229A substitution in E2, was significantly more sensitive to neutralization by sera raised against glycosylated E2 than BICv (NAb titers of 1494 vs. 603 respectively, $p<0.05$, *t*-test). The reactivity to neutralization of the rest of the mutant viruses was similar to BICv (data not shown). The data suggest that mutations at these glycosylation sites may lead to conformational changes in E2 protein that either shield or expose important neutralizing epitopes.

Discussion

Glycosylation of viral envelope proteins influences the immunogenicity and the sensitivity of the virus to neutralizing antibodies. By maintaining the appropriate conformation of proteins, oligosaccharides may hinder proteolytic degradation and with that affect T-cell recognition (Li et al., 2008; Sodora et al., 1991). Removal of glycans from viral envelope proteins has been shown to enhance (Doe et al., 1994; Reitter et al., 1998; Wei et al., 2003), interfere with (Sjolander et al., 1996), or have no effect on their immunogenicity (Bolmstedt et al., 1996). In this study we characterized the role of glycosylation of the three CSFV envelope proteins, analyzed its ability

Table 2
Swine survival and fever response following vaccination of swine with glycosylated and nonglycosylated E^{rns}, E1, and E2 proteins.

Protein	No. of survivors/ total no.	Mean time to death (no. of days ± SD[a])	Fever		
			No. of days to onset (±SD)	Duration (no. of days ± SD)	Max temp, °C (±SD)
E^{rns} nonglycosylated	0/6	13 (1.0)	3.5 (0.5)	4.5 (1.5)	42.1 (0.31)
E^{rns} glycosylated	6/6	–[b]	2.67 (1.5)	3.83 (2.6)	40.8 (0.32)
E1 nonglycosylated	0/2	13.5 (0.5)	4 (0.0)	6 (0.0)	41.6 (0.0)
E1 glycosylated	0/2	14.5 (5.5)	3.5 (0.5)	6.5 (0.5)	41.6 (0.35)
E2 nonglycosylated	0/6	13.5 (0.5)	2.5 (1.5)	9 (2.0)	42 (0.27)
E2 glycosylated	6/6	–	7[c] (1.0)	2.5 (0.5)	41.4 (0.62)

[a] SD: standard deviation.
[b] "–" negative.
[c] Only 2 animals out of total of 6 inoculated animals.

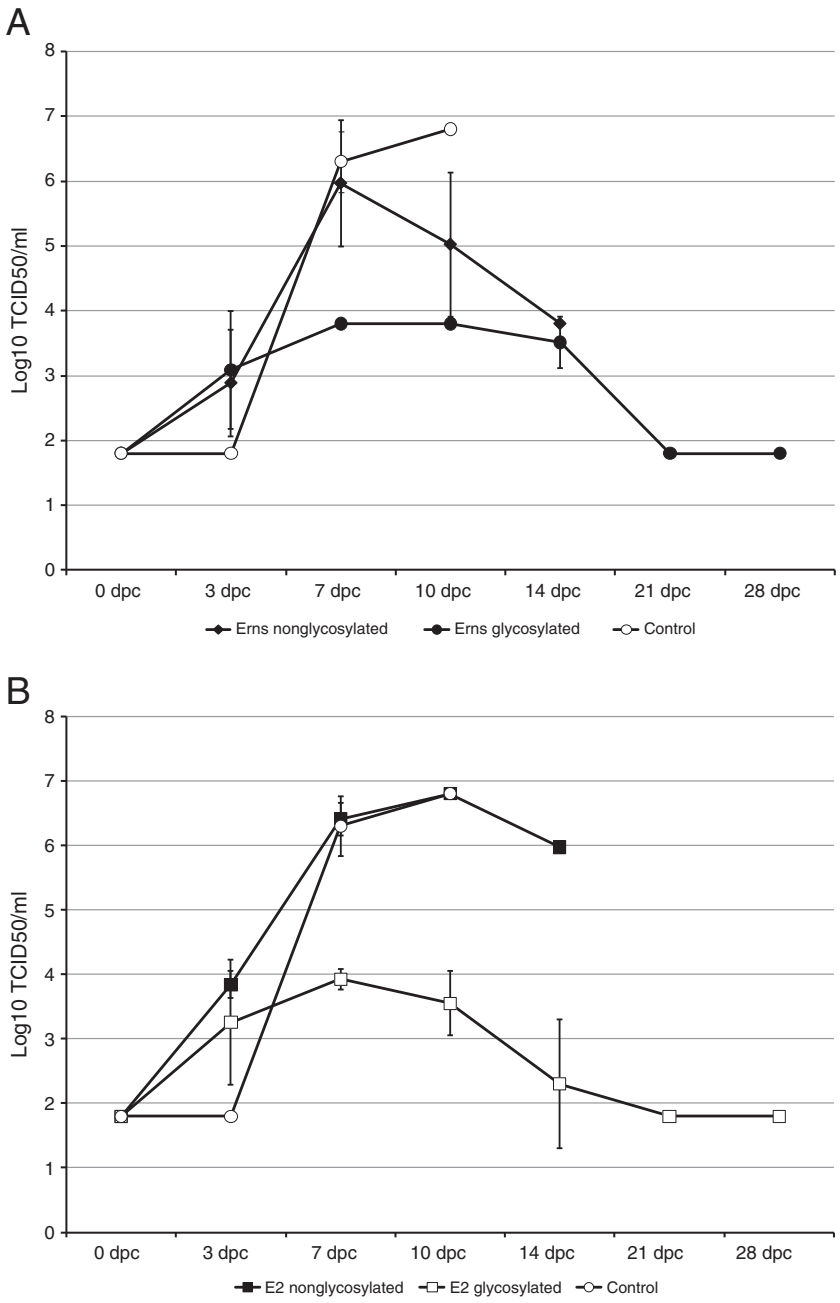

Fig. 3. Means and standard deviations of virus titers in blood from pigs immunized with nonglycosylated (n = 4) and glycosylated (n = 4) forms of E^{rns} (A); and nonglycosylated (n = 4) and glycosylated (n = 4) forms E2 (B) proteins and challenged with BICv. dpc: days post-challenge. Values expressed as log10 $TCID_{50}$/ml. Sensitivity of the assay ≥1.80 log10 $TCID_{50}$/ml.

to induce humoral immune responses, and confirmed its necessity to induce protection in swine against challenge with highly virulent CSFV.

Four conclusions can be drawn from this study: (i) only glycosylated E^{rns} and E2 proteins were able to induce a NA response and protection against CSFV in swine; (ii) E1 glycoprotein was not immunogenic nor protective against CSFV even in its native form and after four immunizations; (iii) purified E^{rns} glycoprotein was immunogenic, inducing an effective protection against CSF in swine even after one immunization; and (iv) the immunogenicity of E^{rns} and E2 glycoproteins is greatly affected by specific glycosylation sites.

(i) Sera from swine inoculated with nonglycosylated forms of the E^{rns} and E2 envelope proteins failed to neutralize CSFV infection of SK6 cells. A similar effect has been previously observed for HIV (Benjouad et al., 1992), where no neutralizing activity was observed in sera from rabbits immunized with deglycosylated forms of major envelope protein gp160. However, in those studies the sera from these rabbits recognized gp160 by RIA; sera from swine immunized with non-glycosylated forms of E^{rns} or E2 did not react with the proteins in competitive ELISA tests. The percentages of inhibition in sera from pigs after four immunizations with nonglycosylated forms of E^{rns} or E2 were similar to those of pre-immune sera. It is likely that modifications induced in these envelope proteins lacking proper post-translational modifications, noticed by complete lack of glycosylation, results in conformational changes that affect exposure of important epitopes that mediate the NA response against CSFV and induce protection against the virus. Here, we found that only glycosylated E^{rns} and

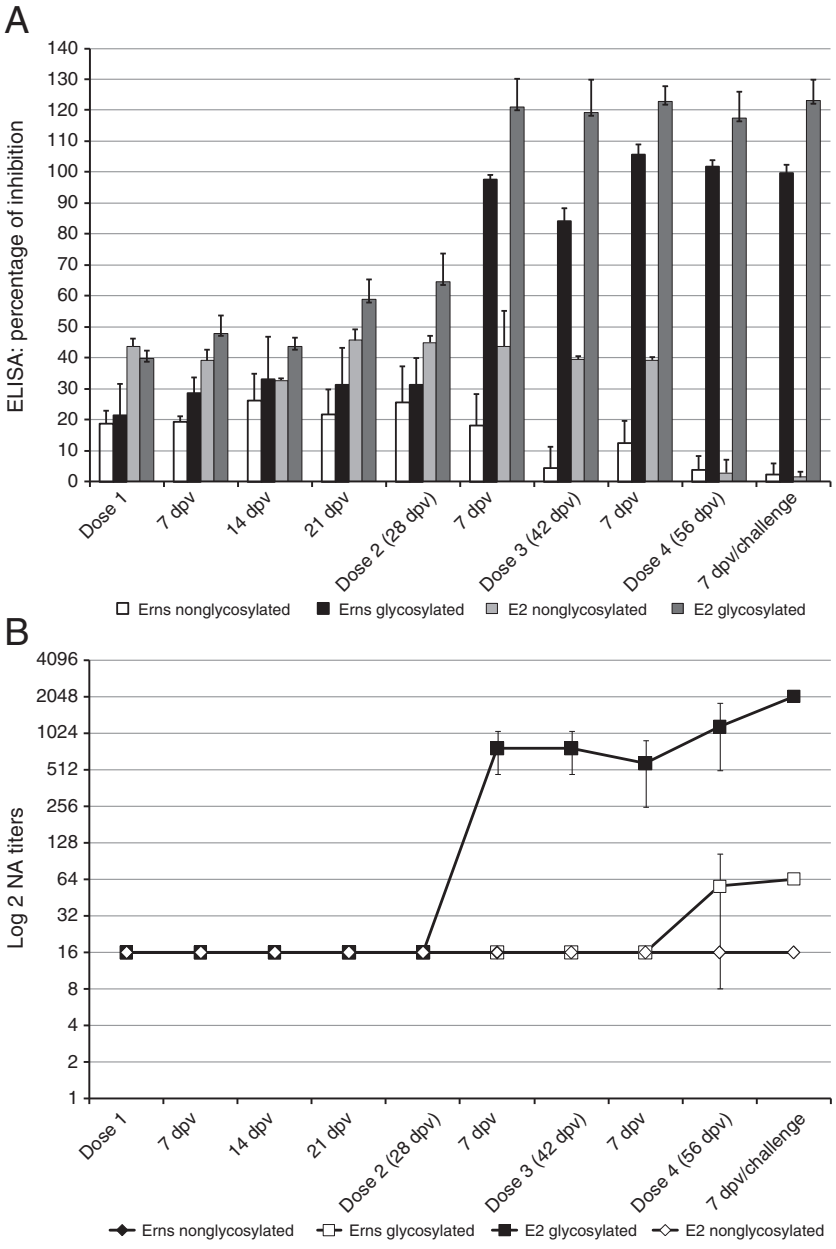

Fig. 4. Antibody response in pigs immunized with nonglycosylated or glycosylated forms of CSFV envelope proteins E^{rns} and E2. Group of pigs, four pigs per group, were inoculated intramuscularly with different forms of the envelope proteins on days 0, 28, 42, and 56; then challenged intranasally with BICv 1 week after last immunization. ELISA antibody titers (A) are expressed as percentage of inhibition (see Materials and methods). NA titers (B) are expressed as log 2 of the reciprocal of the highest serum dilution neutralizing approximately 100 TCID50 of BICV. dpv: days post-vaccination. Error bars correspond to standard deviations.

E2 forms of the proteins can induce the production of antibodies establishing a strong protective response against CSFV.

(ii) The baculovirus-expressed E1 glycoprotein did not induce a detectable NA response in sera from immunized pigs even after administration four times as an oil-in-water emulsion. Under these conditions, E1 did not elicit a protective response since challenged animals succumbed to infection with virulent CSFV. The poor immunogenicity of E1 protein was also observed in animals vaccinated with recombinant competent vaccinia virus that succumbed to CSFV infection when challenged intranasally 5 weeks post-vaccination (Konig et al., 1995). As observed here, those animals did not show detectable antibodies against the protein before challenge, although high antibody titers against the vector were detected. This suggests that E1 protein may not play a significant role in immunity against CSFV.

(iii) Purified E^{rns} protein induced an effective protective response against CSFV. The immunogenicity of E^{rns} was observed first in pigs vaccinated with a replication competent recombinant vaccinia virus that resisted CSFV infection when challenged 5 weeks post-vaccination (Konig et al., 1995). In those animals there were no detectable NA a week before challenge and sera contained only non-neutralizing antibodies detectable by ELISA. Different from the experiments performed here, antigens expressed by recombinant vaccinia viruses are delivered into eukaryotic cells and usually elicit high levels of cytotoxic T lymphocytes (CTL) (Moss, 1991, 1992), leading to effective antiviral activity (Byrne and Oldstone, 1984). However, no CTL epitopes have been reported for E^{rns} protein. We also observed that protected pigs, challenged 21 days after a single vaccination with the E^{rns} protein, did not have detectable NAs or ELISA-detectable

antibodies at the time of exposure to CSFV. Rather, antibodies against E^{rns} were detected by 7 days post-challenge at much higher titers than in mock immunized animals, indicating a boosting effect by the virus infection, whereas NAs were detected later, by 10 days post-challenge. This rapid onset of humoral immunity observed after exposure to CSFV may have played a role in virus clearance and disease progression. The apparent lack of NA titers in protected animals may be the result of the presence of undetectable but still efficacious levels of NAs. Alternatively, protection may be mediated by still undefined immune mechanisms. Nevertheless, these mechanisms are likely to be antibody-mediated (i.e.: antibody-mediated cytotoxicity or antibody-complement-mediated cytotoxicity) since the induction of T-cell mediated mechanisms in animals immunized with purified protein preparations it is less plausible.

(iv) Individual glycosylation sites in E^{rns} and E2 proteins have an effect on the induction of humoral responses after immunization. Glycan moieties may limit the antigenicity of viral envelope proteins and restrict the binding of some antibodies to key epitopes. Contrary to what has been observed for other viruses, removal of individual glycosylation sites from E^{rns} and E2 proteins did not result in enhancement of the antibody response in vaccinated pigs. Rather, vaccination with five of the E2 mutated proteins resulted in lower NA titers (O1, N1, N2, N3, and N4) relative to fully glycosylated protein, suggesting that NA induction depends on the presence of these glycosylation sites. Data from cross-neutralization studies between E2 sera and recombinant glycosylation mutant viruses E2N1v and E2N4v indicate that N1 and N4 sites in the E2 protein affect antigenicity of the protein, an effect compatible with structural changes that affect proper exposure of neutralizing epitopes. Interestingly, N1 and N2 sites are located towards the N-terminus of E2 between two proposed structural units of the proteins, conformed by domains D and A (van Rijn et al., 1994). Both structural units contain neutralizing epitopes. N3 and N4 sites reside in an area of E2 still structurally undefined. Eventually, alteration of glycosylation patterns in E2 might affect exposure of currently unidentified epitopes. Nonetheless all mutated proteins were able to induce a protective immunity after four immunizations. Further mapping of these glycosylation sites will be required to determine what combina-

Table 3
Swine survival and fever response in pigs immunized with different doses of glycosylated Erns proteins and challenged with BICv.

No. of doses (×)	No. of survivors/ total no.	Mean time to death (no. of days ± SD[a])	Fever		
			No. of days to onset (±SD)	Duration (no. of days ± SD)	Max temp, °C (±SD)
3×/challenge at 7 days	2/2	–[b]	3.0 (0.00)	1.0 (0.00)	40.8 (0.77)
3×/challenge at 21 days	2/2	–	–	–	39.7 (0.00)
2×/challenge at 21 days	2/2	–	4.0 (0.00)	4.0 (0.00)	41.6 (0.56)
1×/challenge at 21 days	2/2	–	5.5 (0.71)	2.5 (0.71)	40.7 (0.14)
0×/challenge	0/2	11.5 (2.12)	3.0 (0.00)	9 (1.41)	42.2 (0.35)

[a] SD: standard deviation.
[b] "–" negative.

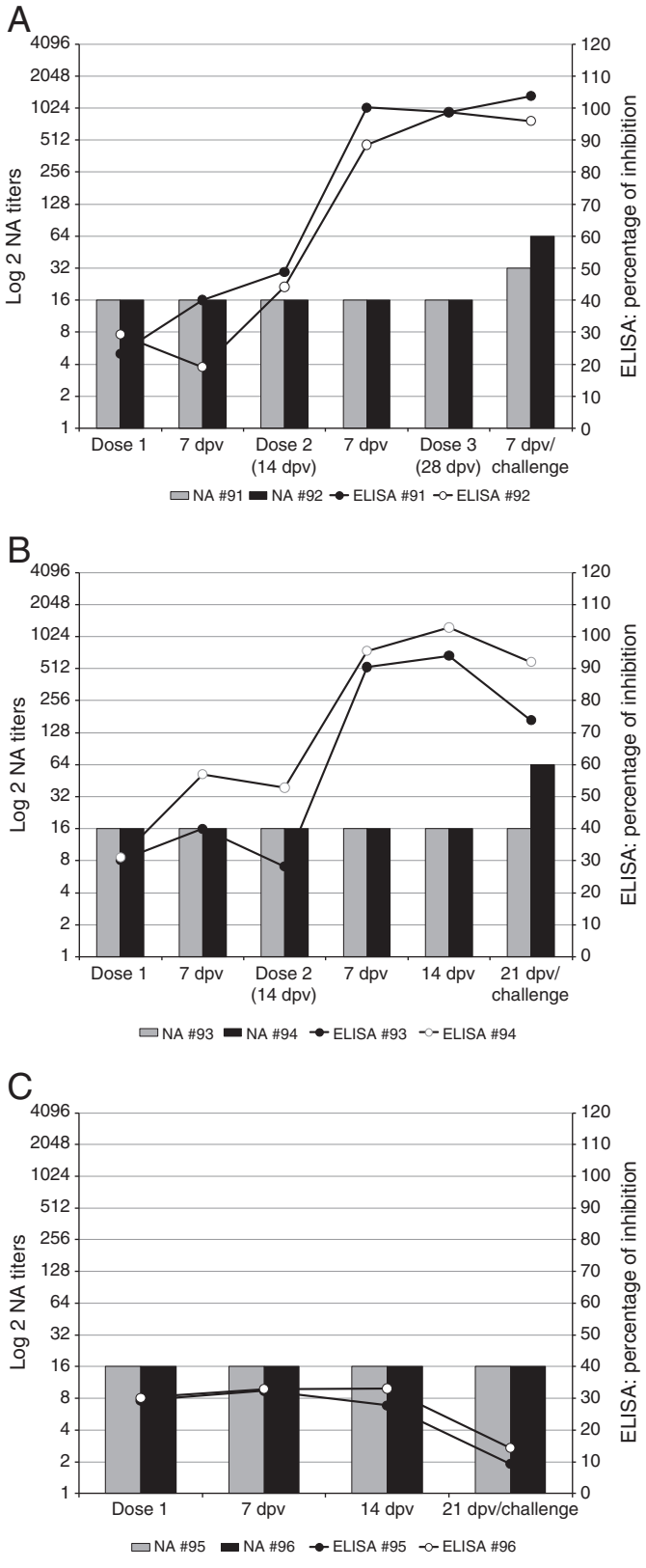

Fig. 5. Antibody response in pigs immunized with different number of doses of glycosylated E^{rns} protein before challenge with BICv. Pigs (n = 2) were inoculated intramuscularly three times at days 0, 14, and 28 and challenged 7 days after last vaccination (A), twice (n = 2) on days 0 and 14 and challenged 21 days after last vaccination (B), or once (n = 2) and challenged 21 days after last vaccination (C). Not shown are animals (n = 2) immunized with 3 doses of E^{rns} challenged at 21 days after last vaccination. Serum samples were collected weekly after the first immunization and antibody titers were detected by ELISA and measured by seroneutralization. NA titers are expressed as log 2 of the reciprocal of the highest serum dilution neutralizing approximately 100 TCID50 of BICv and antibodies detected by ELISA are expressed as percentage of inhibition (see Materials and methods). dpv: days post-vaccination.

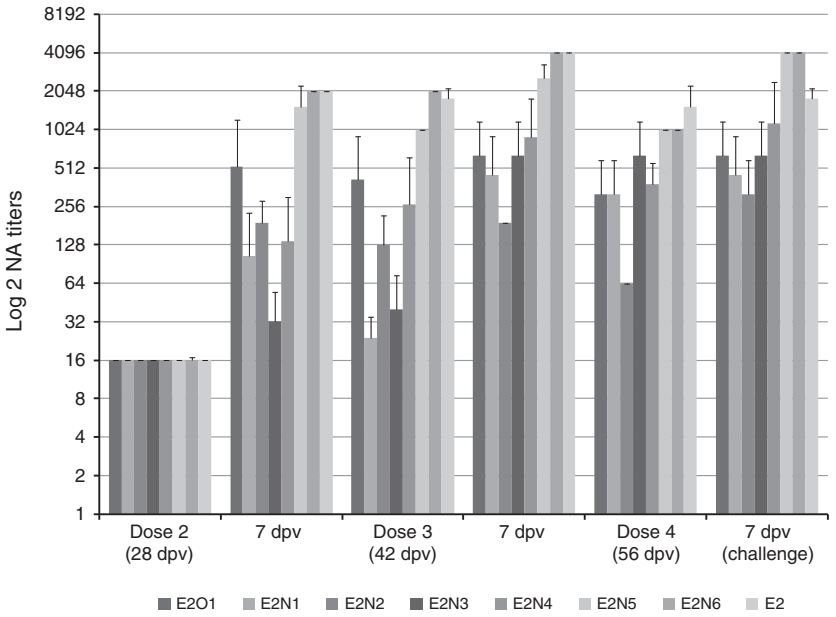

Fig. 6. Development of NAs in pigs immunized with E2 single site glycosylation mutants. Pigs (n = 2 per group) were inoculated intramuscularly with each mutant forms of E2 at days 0, 28, 42, and 56 and challenged 1 week after last immunization. Shown are NA titers from a week previous to seroconversion. NA titers are expressed as log 2 of the reciprocal of the highest serum dilution neutralizing approximately 100 TCID50 of BICv (see Materials and methods). Error bars correspond to standard deviations.

tion of mutations will lead to a complete lack of immunogenicity, as observed here with nonglycosylated forms of the protein. Similarly, vaccination with E^{rns} mutated proteins did not result in enhancement of the antibody response. Removal of individual sites greatly affected the production of NAs, since immunization with glycosylation mutated proteins did not induce detectable NAs, while fully glycosylated E^{rns} induced a NA response detectable by 14 days post-third immunization. In both cases animals were protected against lethal CSFV challenge. Clearly, immunogenicity of E^{rns} is dependent on glycosylation.

Table 4
Swine survival and fever response in pigs immunized with E^{rns} and E2 single site glycosylation mutant forms of the proteins and challenged with BICv.

Protein	No. of survivors/ total no.	Time of death (dpc) (±SD[a])	Time of the onset of fever (dpc) (±SD)	Duration of fever (days) (±SD)	Maximal temperature (°C) (±SD)
E^{rns}	1/1[b]	–	7 (0.00)	1(0.00)	40.1 (0.00)
E^{rns} N1	2/2	–	3 (4.24)	0.5 (0.70)	40 (0.21)
E^{rns} N2	2/2	–	3.5 (4.94)	2 (2.82)	40 (0.28)
E^{rns} N3	2/2	–	3 (2.82)	6.5 (3.58)	41 (1.13)
E^{rns} N4	2/2	–	2.5 (3.53)	1 (1.41)	40 (0.42)
E^{rns} N5	2/2	–	2 (2.82)	3.5 (4.94)	40.4 (0.35)
E^{rns} N6	2/2	–	4.5 (0.70)	3.5 (3.53)	40.3 (0.14)
E^{rns} N7	1/1[b]	–	4 (0.00)	6 (0.00)	41.6 (0.00)
E2	2/2	–	4 (4.24)	3 (2.82)	40.2 (0.21)
E2O1	2/2	–	–	–	39.8 (0.00)
E2N1	2/2	–	2.5 (3.53)	3.5 (4.94)	40.6 (0.91)
E2N2	2/2	–	3.5 (2.12)	8.5 (0.70)	40.8 (0.07)
E2N3	2/2	–	3.5 (4.94)	0.5 (0.70)	40.3 (0.63)
E2N4	2/2	–	6.5 (9.19)	1.5 (2.12)	40 (0.42)
E2N5	2/2	–	7 (9.89)	1.5 (2.12)	40.1 (0.35)
E2N6	1/1[b]	–	–	–	39.6 (0.14)
Control	0/2	11.5 (4.94)	3 (0.00)	9 (4.24)	42 (0.00)

"–" negative.
[a] One of the two animals in each of these groups died at early stages of the immunization period by unrelated causes.
[b] Standard deviation.

In summary, the results presented here indicate that neutralizing epitopes in E^{rns} and E2 proteins are dependent on the presence of glycosylation; most probably, as E2 data suggests, critical glycosylation sites are located within certain domains of this protein (i.e. D/A domains). We show that preventing proper post-translational modification in E^{rns} and E2 that mainly results in lack of glycosylation lead to the synthesis of non-immunogenic proteins that failed to induce protection against CSFV, while removal of single glycosylation sites did not significantly affect the overall immunogenicity of these proteins. We observed that only purified E^{rns} and E2 glycoproteins mediate immunity against CSFV, since glycosylated and nonglycosylated forms of E1 protein failed to induce a detectable antibody response and failed to induce protection in vaccinated pigs. A highly significant observation was that a single dose of purified E^{rns} induces protection against CSFV although the exact mechanism(s) mediating that protection remains uncertain.

It is possible that observations presented here will be useful for designing subunit vaccines against CSFV.

Materials and methods

Cells and viruses

Propagation of recombinant baculoviruses and expression of the envelope proteins were performed in the *Spodoptera frugiperda* derived Sf9 cell line. Sf9 cells were maintained in adherent cultures in Grace's insect cell culture medium (TNM-FH) supplemented with 10% fetal bovine serum and gentamicin (Lonza, Walkersville, MD).

Swine kidney cells (SK6) (Terpstra et al., 1990), free of BVDV, were used to propagate CSFV BICv (Risatti et al., 2005) and glycosylation mutant viruses (Fernandez-Sainz et al., 2009; Risatti et al., 2007; Sainz et al., 2008). SK6 cells were cultured in Dulbecco's minimal essential medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (Lonza).

For titration of CSFV from clinical samples, SK6 cells in 96-well plates (Corning, Lowell, MA) were infected and incubated at 37 °C and 5% CO_2 for 4 days. Viral infectivity was detected using an immunoperoxidase assay with the CSFV monoclonal antibody (mAb) WH303 (Edwards et al., 1991) and the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Titers were calculated according to

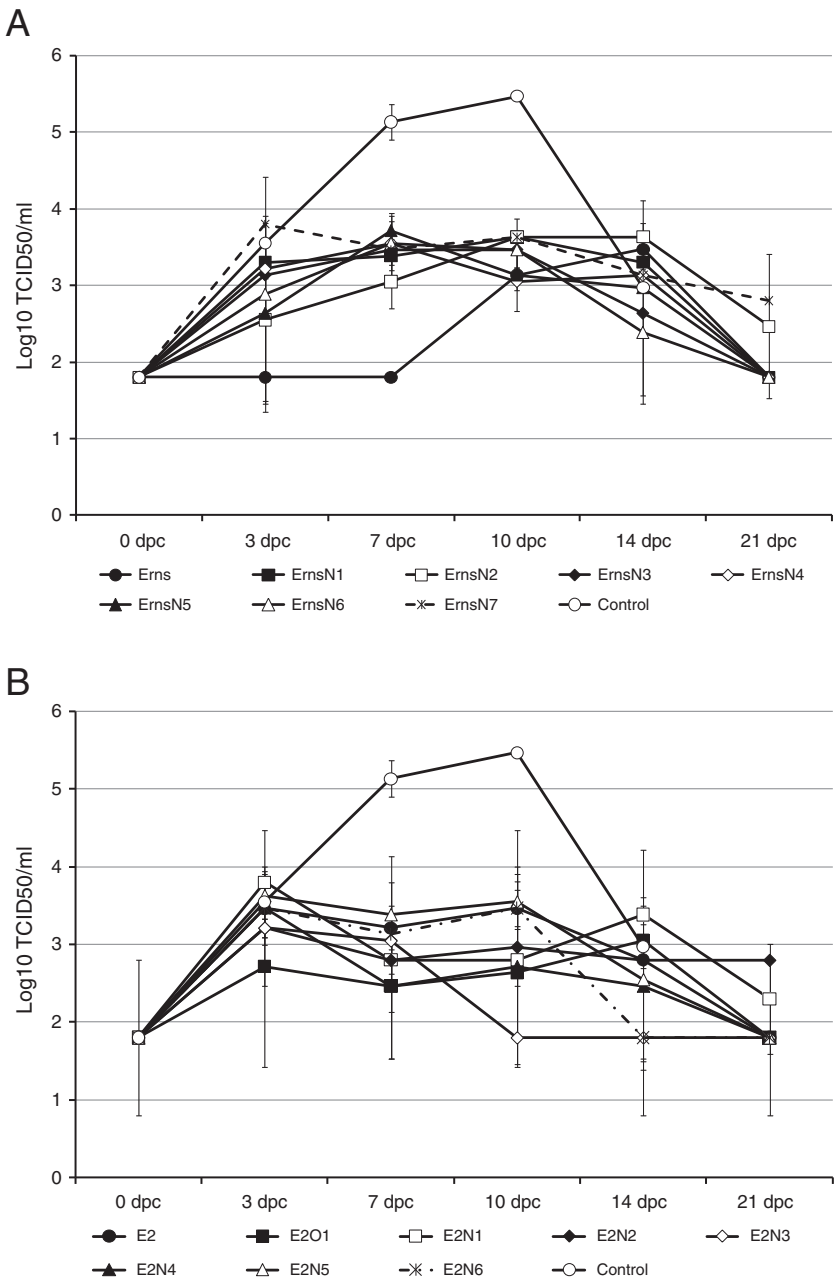

Fig. 7. Average of virus titers in blood from pigs, two pigs per group, immunized with E^{rns} single site glycosylation mutants (A), or E2 single site glycosylation mutants (B) after challenge with virulent CSFV strain Brescia (n = 2). Viremia from one animal only is shown for E^{rns}, E^{rns} N7, and E2N6. dpc: days post-challenge. Values are expressed as log10 $TCID_{50}$/ml. Sensitivity of the assay ≥1.80 log10 $TCID_{50}$/ml. Error bars correspond to standard deviations.

the method of Reed and Muench (1938) and were expressed as 50% tissue culture infective dose ($TCID_{50}$)/ml. As performed, test sensitivity was $\geq \log_{10}$ 1.8 $TCID_{50}$/ml.

Generation of recombinant baculoviruses

Polymerase chain reaction (PCR) was utilized to synthesize CSFV BICv E^{rns}, E1, and E2 genes using specific sets of primers (Table 1). Reverse primers included sequences encoding for six histidine residues (6×-HIS) to allow protein detection and purification by immobilized-metal affinity chromatography. Sets of forward primers with or without sequences encoding for putative CSFV signal peptides (Moser et al., 1996; Ruggli et al., 1995; Rumenapf et al., 1993; van Rijn et al., 1996) were used to synthesize glycosylated and nonglycosylated forms of the three envelope protein genes. The E2 gene was synthesized without its transmembrane region (Hulst et al., 1993) to facilitate its subsequent expression and purification. The resulting PCR fragments were eluted from a 1% agarose gel using the QIAEX II Gel Extraction Kit (Qiagen, Valencia, CA), and cloned directionally into pENTR/D-TOPO entry vector (Invitrogen). To confirm that inserted genes were in frame, entry clones were verified by automatic sequencing.

Single site glycosylation mutated E^{rns} and E2 genes were obtained by site-directed mutagenesis (QuikChange Multi Site-Directed Mutagenesis Kit, Agilent Technologies, Santa Clara, CA) using specific primers (Risatti et al., 2007; Sainz et al., 2008) (Table 1). pENTR/D-TOPO vectors harboring E^{rns} or E2 genes were used as template in those reactions.

Recombinant baculoviruses were produced using the BaculoDirect C-Term expression system (Invitrogen) according to the manufacturer's

instructions. Briefly, recombinant baculoviruses expressing envelope proteins were constructed by an attL-attR recombination reaction between entry clones and BaculoDirect Linear DNA containing the Herpes Simplex Virus thymidine kinase gene located between recombination sites for negative selection with ganciclovir. Sf9 cells were transfected using Cellfectin (Invitrogen) and subsequently propagated as recommended in the protocol provided by the manufacturer. Cells were grown until signs of infection were observed (6–8 days after transfection). The resulting stock (P1) was harvested and amplified once by infecting confluent Sf9 cells to obtain a high-titer viral stock (P2).

Detection and purification of recombinant proteins

Sf9 cells (about 1.5×10^6/ml) were infected at MOI = 5 or 10 with recombinant baculoviruses and cells and culture media were harvested after incubation at 27 °C for 5 to 7 days. Cultures were centrifuged at $2000\times g$ for 5 min at 4 °C and the cell pellet was washed twice with phosphate-buffered saline (PBS) followed by resuspension in RIPA buffer (Sigma Aldrich, St. Louis, MO). After incubation for 10 min on ice, preparations were clarified by centrifugation at $16{,}100\times g$ for 10 min. Expression of the recombinant envelope proteins was verified by Western blot analysis. Briefly, protein lysates were separated under reducing conditions on a 10% NuPage Novex Bis–Tris gel (Invitrogen) by a discontinuous SDS-PAGE system and transferred to polyvinylidene fluoride (PVDF) membrane (Invitrogen). Membranes were blocked, and then incubated overnight with anti-his C-term antibody (Invitrogen) at a dilution of 1:5000. Membranes were washed and incubated with goat anti-mouse IgG antibody conjugated with alkaline phosphatase (WesternBreeze Chemiluminescent Detection Kit, Invitrogen). Envelope proteins were detected using CDP-star chemiluminescent substrate provided with the kit and by exposing to X-ray films (Kodak X-OMAT LS film, Kodak, Rochester, NY).

For purification, protein lysates were applied to HisPur Cobalt Resin Columns (Thermo Fisher Scientific, Rockford, IL) and purified following the manufacturer's instructions. The collected fractions were analyzed by Western blotting as described above and Coomassie blue staining (Simply Blue SafeStain, Invitrogen).

As control for deglycosylation of envelope proteins, glycosylated envelope proteins were treated with peptide N-Glycosidase F (PNGase F, New England Biolabs, Ipswich, MA) following the manufacturer's directions. Briefly, infected cell extracts were denatured at 100 °C for 10 min in glycoprotein denaturing buffer (New England BioLabs). The reaction mixture was placed on ice for 5 min, and then digested with PNGase F for 1 h at 37 °C in the presence of 1% NP-40.

Protein concentration was determined by the BCA Protein Assay (Thermo Fisher Scientific) against bovine serum albumin standard, by measuring OD at 595 nm in a NanoDrop (NanoDrop Technologies, Wilmington, DE).

Animal experiments

Eighteen to twenty kiliograms, 2 to 3 months old, commercial breed pigs were used in all these experiments. In all cases, pigs were inoculated with purified envelope proteins mixed with Sigma Adjuvant System (Sigma-Aldrich, St. Louis, MO) to obtain an oil-in-water emulsion according to instructions given by the manufacturer. One milliliter of each inoculum containing ~50 μg of purified protein was injected via intramuscular route into the neck of animals. Mock-vaccinated animals received adjuvant alone. After immunization pigs were challenged intranasally (IN) with 10^5 $TCID_{50}$ of highly virulent BICv.

For initial screening, pigs (n = 14) were allocated randomly into 6 groups (n = 2). A mock-vaccinated control group (n = 2) was also included. Pigs were inoculated with nonglycosylated or glycosylated forms of E^{rns}, E1, or E2 proteins and boosted with the same dose 28, 42, and 56 days later. Blood samples were collected from each animal weekly starting from the day of first inoculation. Pigs were challenged 1 week after the last inoculation. After challenge, pigs were observed daily for 28 days for manifestation of CSF clinical signs (anorexia, depression, purple skin discoloration, staggering gait, diarrhea, and cough). Body temperatures were recorded daily throughout the experiment.

For confirmatory purposes, pigs (n = 18) were randomly allocated into one of four vaccination groups (n = 4) or a mock-vaccination control group (n = 2). Pigs were inoculated with nonglycosylated or glycosylated forms of E^{rns} or E2 proteins (vaccinated groups) or non-vaccinated (control group). Protein concentrations, route of inoculation, adjuvant formulations, vaccination schedule, and challenge were performed as described above. Blood, serum, nasal swabs, and tonsil scrapings were collected from each pig at 0, 3, 7, 10, 14, 21, and 28 days post-challenge (dpc).

The protective efficacy of glycosylated E^{rns} protein was further assessed in swine. Pigs (n = 10) were allocated into 4 vaccine groups (n = 2) and 1 non-vaccinated control group (n = 2). Pigs were inoculated as described above. Two vaccine groups received 3 doses of glycosylated E^{rns} at 2-week intervals (days 0, 14, and 28) and were challenged at 7 and 21 days post-third inoculation, respectively. A third vaccine group received 2 doses of glycosylated E^{rns} at 2-week interval (days 0 and 14) and was challenged at 21 days post-second inoculation. The fourth vaccine group received 1 dose of glycosylated E^{rns} (day 0) and was challenged at 21 days post-inoculation. Serum samples were obtained weekly after vaccination.

To assess the immunogenicity of single site glycosylation E^{rns} and E2 mutated proteins, pigs (n = 34) were randomly allocated into sixteen vaccinated groups (n = 2), and a mock-vaccinated control group (n = 2). Seven vaccinated groups received single site glycosylation mutated E^{rns} proteins (N1, N2, N3, N4, N5, N6, and N7) (Figs. 1 and Supplementary Fig. S1A). Seven vaccinated groups received single site glycosylation mutated E2 proteins (O1, N1, N2, N3, N4, N5, and N6) (Figs. 1 and Supplementary Fig. S1B). Two vaccinated groups received glycosylated E^{rns} and E2 proteins, respectively. The control group was mock vaccinated. Animals were inoculated with these proteins at days 0, 28, 42, and 56 and then challenged 1 week after the last vaccination.

Antibody detection

Serum samples were analyzed for the presence of CSFV E^{rns} specific antibodies using commercial ELISA kits: IDEXX CHEKIT CSF MARKER ELISA Test Kit (Idexx Laboratories, Westbrook, ME) for detection of antibodies against E^{rns} and detection of anti-E2 antibodies was performed with IDEXX CSF SERO Antibody ELISA Test Kit (Idexx Laboratories). ELISA test kits were used according to the protocols provided by the manufacturer.

Seroneutralization and cross-neutralization assays were performed with heat-inactivated serum samples (56 °C for 30 min). Two-fold serial dilutions of serum were prepared in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and mixed with equal volumes of BICv or glycosylation mutant viruses (Risatti et al., 2007; Sainz et al., 2008) containing 10^2 $TCID_{50}$. Serum-virus mixtures were incubated for 1 h at 37 °C and then transferred to 96-well flat-bottom tissue culture plates (Corning) followed by addition of SK6 cells (1×10^4 per well). Plates were incubated at 37 °C and 5% CO_2 for 4 days. Supernatant was then removed from each well and the cells were fixed with methanol–acetone (50% vol./vol.) solution and air-dried. Plates were stained by immunoperoxidase assay using the Vecstatin ABC Kit, Vector Laboratories, Burlingame, CA following manufacturer's instructions. Neutralizing antibody titers were expressed as the reciprocal of the highest two-fold serum dilution neutralizing BICv (Reed and Muench, 1938).

Supplementary materials related to this article can be found online at doi:10.1016/j.virol.2011.08.025.

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